

Antibody responses of dengue fever patients to dengue 2 (New Guinea C strain) viral proteins

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Abstract

The present study was undertaken to investigate the antibody responses of dengue fever (DF) patients to specific dengue virus proteins. Partially purified dengue 2 New Guinea C (NGC) strain virus was used as antigen. Under the present experimental protocols, it was observed that almost all DF patients' sera had detectable presence of antibodies which recognize the dengue 2 envelope (E) protein. The convalescent-phasesera especially had significant detectable IgG, IgM and IgE against the protein. In addition, IgGs specific against the NS1 dimer and PrM were also detected. Antibody against the core (C) protein, however, was not detectable in any of the DF patients' sera. The substantial presence of IgG against the PrM in the convalescent-phasesera, and the presence of IgE specific for the E, reflect the potential importance of these antibody responses in the pathogenesis of dengue.

Key words: Antibody, core, envelope, dengue, dengue fever, NS1

INTRODUCTION

Dengue viruses are positive* single-stranded RNA viruses belonging to the family *Flaviviridae*.¹ The virus has been noted to infect and cause a wide spectrum of clinical presentations from asymptomatic infection or mild self-limited febrile illness to life-threatening dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). Infection by the virus is endemic in many tropical and subtropical countries and is rapidly spreading beyond the traditional boundary of the dengue belt areas. In Malaysia, it is estimated that the median incidence rate of dengue infection is at least 27 cases per 100,000 population with some areas reporting up to 132 cases per 100,000 population.² The loss of manpower productivity due to dengue virus infection could amount to millions of ringgit a year. Thus, it is only natural that concerted efforts be taken to overcome the infection if not to completely eradicate it. To this end, a vaccine against dengue viruses is much desired.

The development of an effective and safe vaccine against dengue viruses, however, has been hampered by the presence of cross-reacting serotypes of the dengue viruses. The four serotypes; dengue 1, 2, 3, and 4 are known to elicit host immune responses which may in certain circumstances actually enhance dengue

virus infectivity.^{3,4,5} The present study was undertaken to investigate the antibody responses of dengue fever (DF) patients to the dengue 2 NGC virus antigens fixed onto nitrocellulose membrane.

MATERIALS AND METHODS

Cell culture and virus preparation

Mosquito cells derived from *Aedes albopictus*, C6/36, were used in this study. Cells were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum (PAA Laboratories, Lirz, Austria) in 180 cm² plastic tissue culture flasks (Nunc, Roskilde, Denmark). Confluent C6/36 cells were infected with the New Guinea C (NGC) strain of dengue-2 virus (American Type Culture Collection, Rockville, MD, USA) to give an estimated multiplicity of infection (MOI) of about 3-5 plaque forming unit (PFU) per cell. After about 7-10 days post-infection (PI) or when more than 90% of the infected cells have shown the cytopathic effects (CPE), cell cultures were frozen at -70°C. Crude virus inoculum was prepared by freeze-thawing the infected cell cultures and centrifuging at 800 x g to remove cell debris. The cell culture supernatant obtained following an additional centrifugation at 40,000 x g was then used for infection. Virus was partially purified by

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overlaying the supernatant on-top of a 50% D-sorbitol cushion. The samples were then centrifuged at **105,000 x g** for 2 hours at 4°C and virus fractions were collected from the interphase. The fractions were then diluted in homogenization buffer (10 mM **Tris-HCl**, pH 7.5, 150 mM **KCl**, 2 mM **CaCl₂**, 2 mM **MgCl₂**) and centrifuged at **105,000 x g**. The resulting virus **pellet** was resuspended in homogenization buffer and total protein concentration of the virus suspension was determined using the Micro-BCA protein assay system (Pierce, Rockford, IL, USA). Anti-proteases **aprotinin** (1 **µg/ml**), leupeptin (1 **µg/ml**), and pepstatin A (2 **µg/ml**) were added to the virus suspension following determination of the protein concentration. Samples were kept frozen at -70°C until needed.

Patients sera

Sera of confirmed DF cases were **kindly** provided by Prof. Lam Sai Kit (Department of Medical Microbiology, Universiti Malaya, Malaysia). These sera were obtained with consent from DF patients seen at the University Hospital (UH), University of Malaya, Kuala Lumpur, Malaysia and the neighboring hospitals. Confirmation of dengue virus infection was done by staff at the **UH/UM Arbovirus** Diagnostic Unit using hemagglutination-inhibition (HI) test, **IgM** capture ELISA, and virus isolation using tissue cultures and mosquito larvae following the standard WHO protocols.⁶

Viral protein separation and immunodetection

Partially purified dengue 2 NGC strain virus was separated by non-denaturing 10% **polyacrylamide** gel electrophoresis (PAGE) with only 0.1% sodium dodecyl sulphate (SDS). Samples containing only 0.1% SDS were not heat-treated and no reducing agents were used. Following electrophoresis, proteins were electrotransferred onto nitrocellulose membrane (MSI, Westborough, MA, USA) and non-specific protein binding was blocked using blocking buffer (100 mM Tris base, pH 7.5, 150 mM **NaCl**) containing 5% skim milk. Following several washings, the membrane was placed in the Mini-PROTEAN II Multiscreen Apparatus (Bio-Rad Laboratories, Hercules, CA, USA) and incubated with sera of **confirmed** DF patients diluted in blocking buffer at **1:20**, **1:100**, and **1:1000** dilution for detection of **IgE**, **IgM**, and **IgG**, respectively. Incubation was done overnight at 4°C with continuous gentle shaking.

The presence of dengue 2 NGC specific antibody **isotypes** was detected using biotinylated **anti-human** heavy chain immunoglobulin monoclonal antibodies (**Kirkegaard & Perry** Laboratories (KPL), Gaithersburg, MD, USA), alkaline phosphatase conjugated streptavidin (**SA-AP**; Pierce, Rockford, IL, USA), and developed using **NBT/BCIP** reagents (KPL, Gaithersburg, MD, USA). During the course of the study we found that by preincubating the biotinylated antibodies with SA-AP, the background and non-specific binding was reduced substantially in comparison to sequential addition of the reagents. This method was used throughout the investigation.

Monoclonal antibodies

Monoclonal antibody against dengue 2 envelope (E) protein was prepared from the culture fluid of the **3H5-1** hybridoma cells (ATCC, Rockville, MD, USA) using anionic exchange column and the **ConSep** LC 100 perfusion chromatography system (**PerSeptive** Biosystems, University Park, MA, USA). Ascitic fluids containing monoclonal antibodies specific to dengue 2 **NS1** and C were provided by Dr. Jane Cardosa (UNIMAS, Sarawak, Malaysia) and Dr. John Aaskov (Queensland University of Technology, Brisbane, Australia), respectively. Biotinylated goat anti human γ , μ , and ϵ monoclonal antibodies were purchased from KPL (Gaithersburg, MD, USA).

Reagents and chemicals

Tissue culture media, reagents and chemicals were purchased from Gibco BRL Life Technologies (Grand Island, NY, USA). Antiproteases pepstatin A, leupeptin, and aprotinin were obtained from Sigma Chemical Company (St. Louis, MO, USA). The broad range **prestained** and biotinylated protein markers used throughout the study were purchased from New England **BioLabs** (Beverly, MA, USA). The protein markers were used as recommended by the manufacturers. The protein markers were treated with **DTT** and the samples were boiled prior to loading into the 0.1% SDS-PAGE.

RESULTS

Recognition of dengue 2 NGC viral proteins by pooled DF patients' sera

In an initial investigation, pooled confirmed DF patients' sera with a predetermined HI titer of $1: \geq 1,280$ were used for detection of dengue 2 NGC viral proteins. The viral proteins were

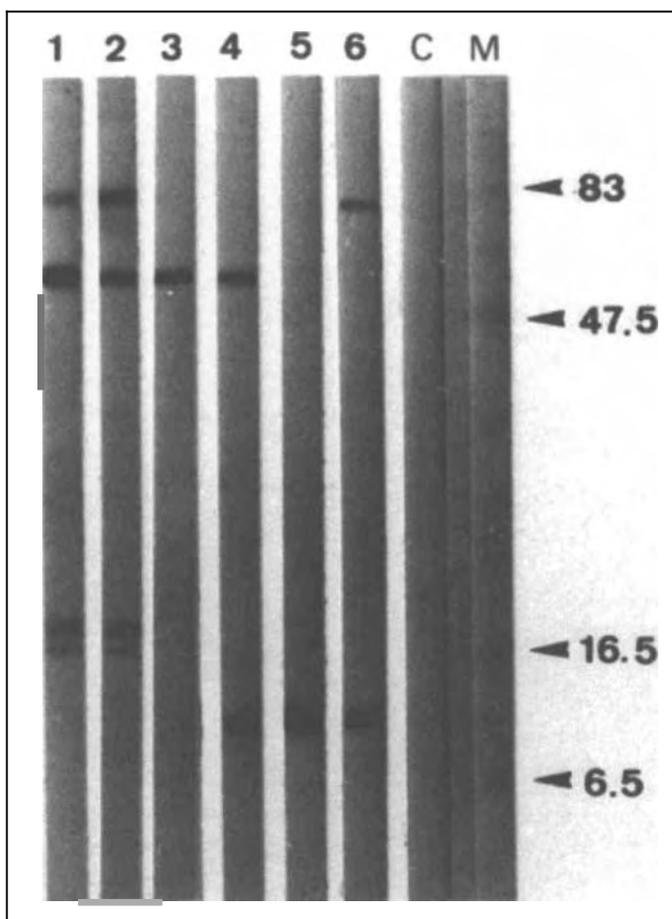


FIG. 1: Detection of dengue 2 NGC viral proteins by immunoblotting. Dengue 2 NGC virus-infected cell lysate was prepared as described in the Materials and Methods. Proteins were separated by PAGE under non-denaturing condition and then electrotransferred onto nitrocellulose membranes. The presence of specific dengue 2 NGC viral antigens was detected using pooled confirmed DF patients sera with secondary infection (lane 1), mouse **hyperimmune** sera (lane 2), dengue 2 E specific monoclonal antibody (lane 3), combination of dengue 2 E and C specific monoclonal antibodies (lane 4), C specific monoclonal antibody (lane 5), and C and NS1 specific monoclonal antibodies (lane 6). Pooled **confirmed** dengue negative sera were used as control (lane C). The blot was developed using alkaline phosphatase-conjugated **anti-human IgG** (lanes 1 and C), **anti-mouse IgG** (lanes 2 to 6) and **NBT/BCIP** substrate. The protein molecular weights shown are in **kilodalton**.

separated by non-denaturing PAGE and electrotransferred onto nitrocellulose membrane. The presence of **IgG** specific against the dengue 2 NGC antigen was detected using biotinylated anti-human **IgG** heavy chain monoclonal antibody. It was observed that pooled secondary DF patients sera had **IgG** which recognized at least four major dengue 2 NGC viral proteins of about 82, 57, 17, and 16 kD (Fig. 1, lane 1). These proteins were detectable also using mouse hyperimmune sera (Fig. 1, lane 2). The approximate molecular weights of these proteins were determined using denatured protein markers

electrophoresed concurrently with the native samples under a non-denaturing condition (see Materials and Methods). The 57 and 82 kD proteins were recognized also by the monoclonal antibodies specific for dengue 2 NGC E and NS1, respectively (Fig. 1, lanes 4 and 6), suggesting that the 57 and 82 kD proteins recognized by the pooled sera were likely to be the E and NS1 dimer of the dengue 2 NGC virus. The monoclonal antibody specific against dengue 2 NGC C, however, recognized a protein of about 12.5 kD (Fig. 1, lanes 4, 5, and 6) which apparently was not detectable using the pooled

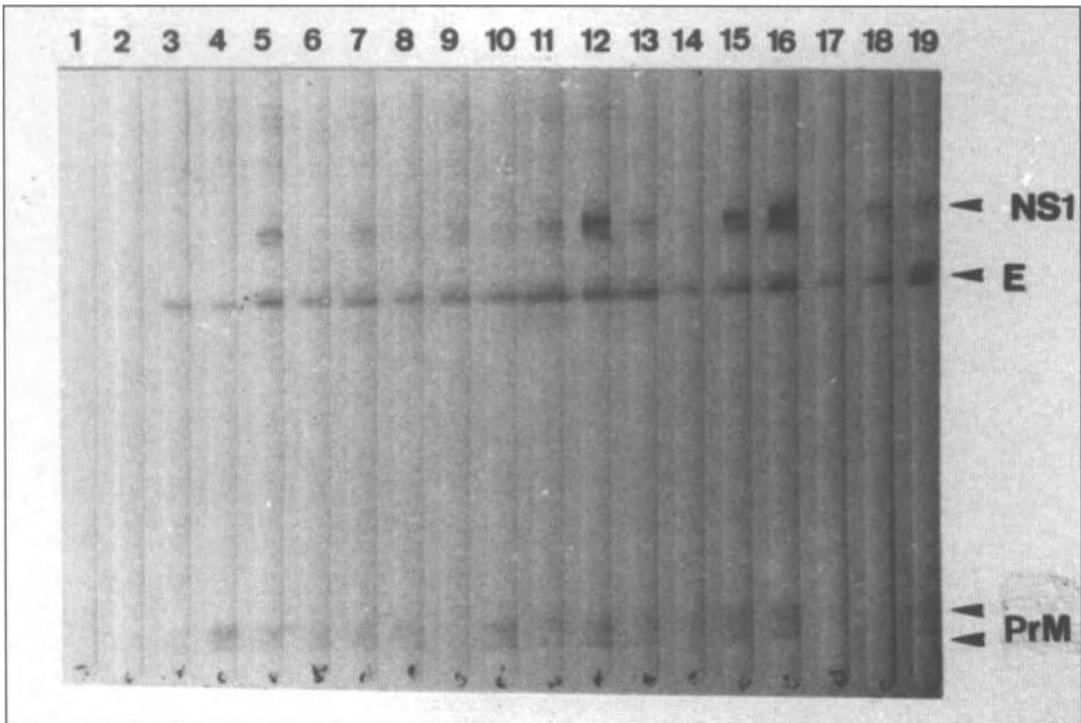


FIG. 2: Detection of IgG against dengue 2 NS1 viral proteins. Sera of confirmed dengue negative (lanes 1 and 2) and DF patients with secondary infection (lanes 3 to 19) were screened for the presence of dengue 2 virus specific IgG using the Mini-PROTEAN II Multiscreen Apparatus (BioRad, USA). Proteins were prepared, separated and immunoblotted as described in Fig. 1. The presence of dengue specific IgG in the patients' sera was detected using biotinylated anti human IgG monoclonal antibody, alkaline phosphatase-conjugated streptavidin, and NBT/BCIP substrate.

secondary DF patients' sera and mouse hyperimmune serum (Fig. 1, lanes 1 and 2). The identity of the 17 and 16 kD proteins recognized by the pooled DF patients' sera could not be readily ascertained. Nevertheless, since these proteins were larger than the C and were most likely to be one of the virus structural proteins, in this report they were referred to as the PrM. The smaller protein could be the remaining Pr following cleavage of the PrM.

Screening of DF patients' sera for IgG that recognizes dengue 2 NS1 viral proteins

Even though at least 4 major dengue 2 NS1 proteins were recognized by the pooled sera, it was not certain if individual DF patient serum would also recognize the 4 proteins. To investigate this possibility, sera of seventeen serologically confirmed DF patients with secondary infection (HI, $1: \geq 1,280$; IgM capture ELISA negative) were evaluated for the presence of IgG specific against the dengue 2 proteins. It

was noted that all DF patients' sera had IgG which recognized the 57 kD dengue 2 E protein (Fig. 2). Detectable presence of IgG against the NS1 dimer and PrM, on the other hand, varies from patient to patient. At least 64% (11/17) of the patients had IgG against the NS1 and only about 47% (8/17) had IgG against both the PrM and NS1. Based on these results, it was apparent that only the dengue 2 E protein was recognized by all DF patients' sera.

Recognition of dengue 2 NS1 proteins by acute- and convalescent-phase sera of DF patients

The lack of consistent detectable immune responses against other dengue virus proteins, beside the E, could be attributed to the clinical stages of infection of the different patients. One possibility was that the secondary infection sera provided (randomly) were obtained from patients with either acute- or convalescent-phase of infection, with sera at the later phase producing significantly more antibodies recognizing other

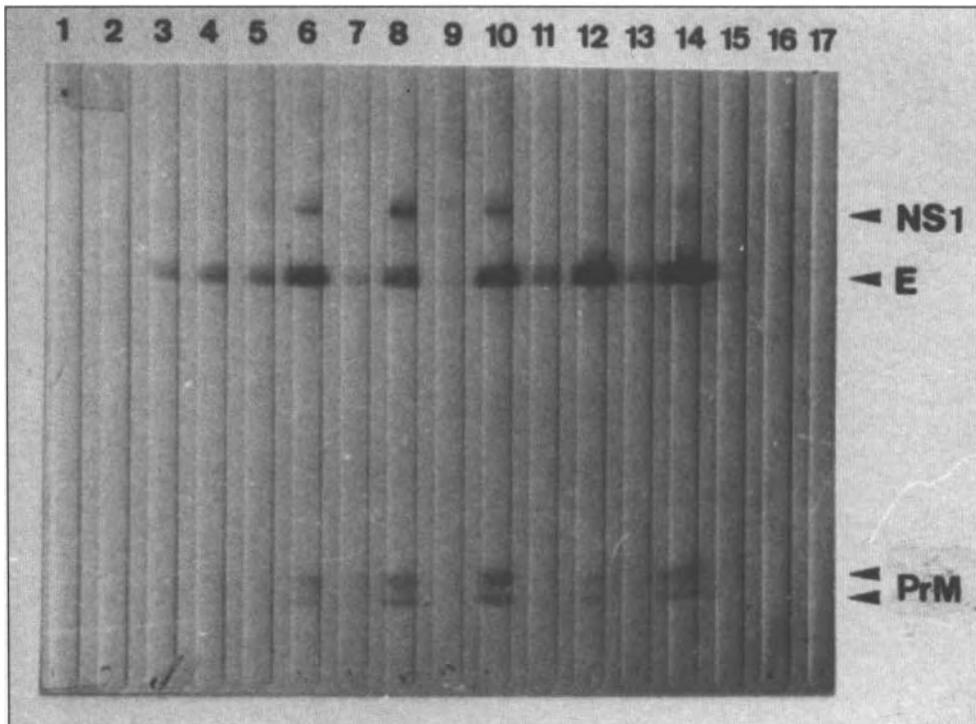


FIG. 3: Detection of IgG specific against dengue 2 NS1, E, and PrM viral proteins. Paired acute (lanes 1,3,5,7,9,11,13,15) and convalescent (lanes 2,4,6,8,10,12,14,16) phase sera of two confirmed dengue negative (lanes 1 and 2; 15 and 16), one DHF patient (lanes 3 and 4) and five DF patients (lanes 5 to 14) were screened for the presence of dengue specific IgG as described in Fig. 2. The negative patients sera and the samples to which no serum was added (lane 17) did not show any detectable proteins. Dengue 3 virus was isolated from sera of two DF patients (lanes 7 and 8; 13 and 14), whereas dengue 2 virus was isolated from all other patients' sera

viral proteins beside the E. To investigate this possibility, acute- and convalescent-phase sera of DF patients with secondary dengue virus infection were obtained and evaluated following a similar protocol as described above. Similar to the earlier findings, results obtained from this investigation showed that almost all DF patients' sera (acute- and convalescent-phase sera) showed detectable presence of IgG against dengue 2 NS1, E (Figure 3). The acute-phase sera of several patients (Fig. 3, lanes 7 and 9), however, initially showed almost undetectable presence of IgG against the E but the convalescent-phase sera which were taken 5 and 7 days later, respectively, showed substantial presence of the IgG against it (Fig. 3, lanes 8 and 10). IgG against dengue 2 NS1, E was detectable also in the convalescent-phase sera from which dengue 3 virus was isolated (Fig. 3, lanes 8 and 14). Under our experimental conditions, it was noted that IgG against other dengue 2 NS1, E proteins (detected with pooled DF sera) were only barely

detectable or undetectable in all the acute-phase sera (Fig. 3, lanes 3, 5, 7, 9, 11, 13). On the other hand, substantial presence of IgG against the NS1 dimer and the PrM was noted in most of the convalescent-phase sera (Fig. 3, lanes 6, 8, 10, 12, and 14), suggesting that IgG against these proteins were highly detectable during the convalescent-phase of secondary dengue virus infection. It was noted also that the only convalescent-phase serum available from a DHF patient, did not have IgG that recognize the NS1 and PrM (Fig. 3, lane 4). Furthermore, the convalescent-phase sera from which dengue 3 was isolated also showed substantial presence of IgG against the NS1 and PrM of dengue 2 NS1, E, suggesting that there were cross reacting antibodies in the serum (Fig. 3, lanes 8 and 14).

IgM responses to dengue 2 NS1, E, and PrM viral proteins by sera of confirmed DF patients

The presence of IgM against dengue 2 NS1, E, and PrM viral proteins in sera of DF patients was

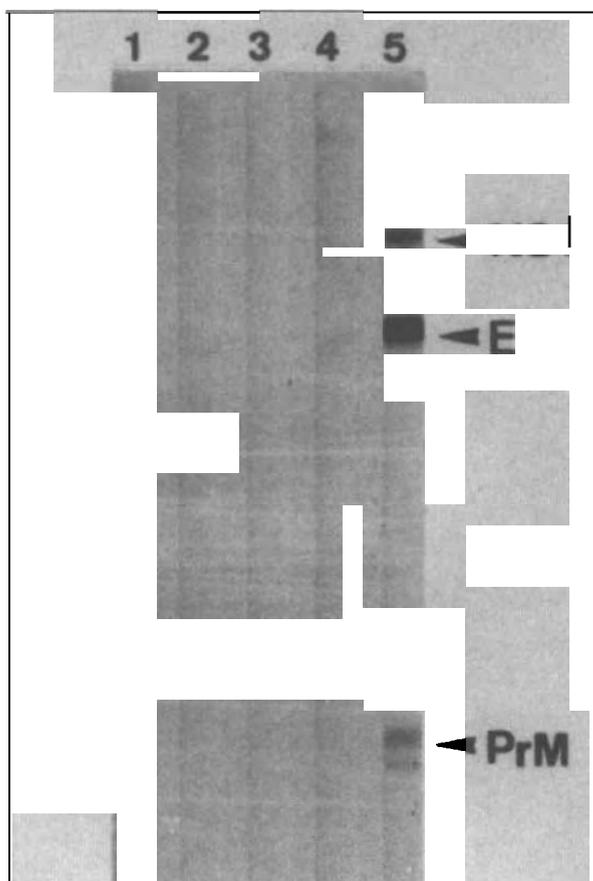


FIG. 4: Comparison between pooled sera of primary and secondary dengue virus infection for detection of dengue 2 proteins. Pooled IgM positive patients sera with low ($1: < 20$) IgG (lanes 1 and 2) were used for detection of IgM (lane 1) and IgG (lane 2) specific for dengue 2 viral proteins. The results were compared to a similarly performed screening where pooled DF positive sera with low to negative IgM ELISA and high HI ($1: \geq 1280$) were used (lanes 4 and 5). Pooled sera of confined negative patients were used as control for both detection of IgM and IgG (lane 3). Screening for the antibodies specific for the dengue virus antigens were performed as described in Figs. 1 and 2.

investigated using biotinylated anti-human IgM monoclonal antibodies. Initially, pooled sera of DF patients with primary infection (IgM capture ELISA positive, HI < 20) were used. Using these sera, IgM specific against the dengue 2 NGC E and NS1 was detectable (Fig. 4, lane 1). The presence of IgG specific against the E, on the other hand was barely detectable (lane 2). In contrast, pooled DF patients' sera with secondary infection (IgG, $1: \geq 1,280$; IgM capture ELISA negative), had a barely detectable presence of IgM specific against the E (Fig. 4, lane 4) but a substantial presence of IgG against the E, NS1, and the PrM (Fig. 4, lane 5). When 16 randomly picked IgM capture ELISA positive DF patients sera were evaluated, about 81% (13/16) showed detectable presence of IgM against the dengue 2

E (Fig. 5). None of the sera, however, had detectable presence of IgM against the NS1 or the PrM.

When comparison was made between the DF patients' antibody responses during acute- and convalescent-phase of the infection, it was revealed that most acute-phase sera did not show significant detectable presence of the dengue 2 NGC specific IgM (Fig. 6). The convalescent-phase sera on the other hand, had detectable presence of IgM against mainly the dengue 2 E and only one patient's serum showed detectable presence of IgM against the NS1 (Fig. 6, lane 6). Similar to the earlier observations, it was noted also that none of the patients sera showed detectable presence of IgM against the PrM.

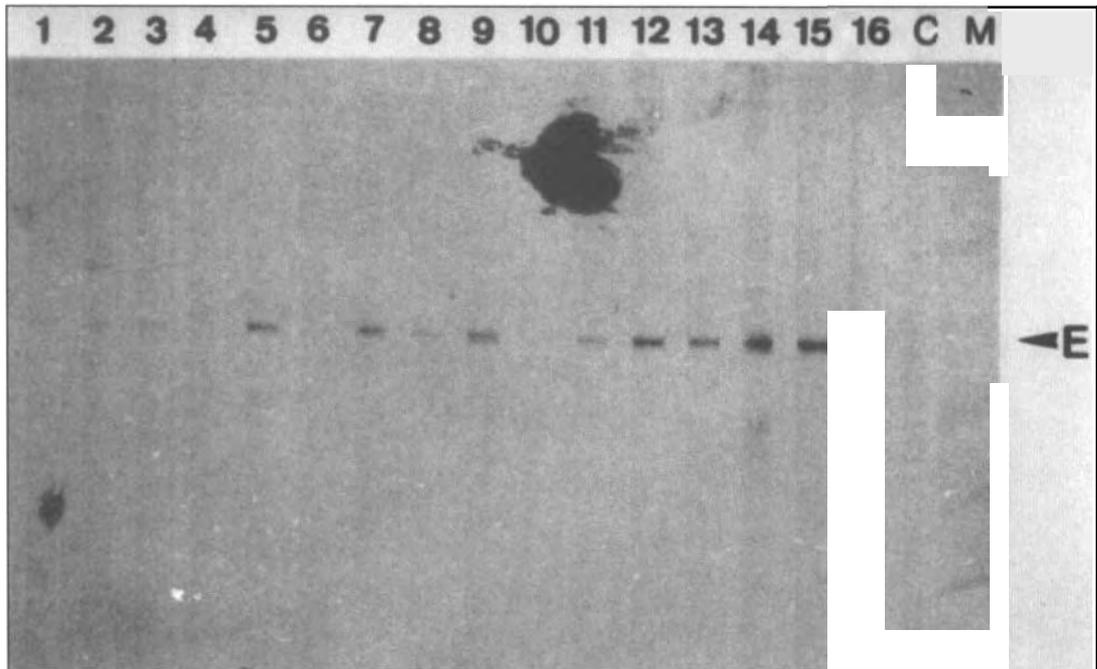


FIG. 5: IgM responses to dengue 2 NS1 viral proteins. Sera of confirmed DF patients (lanes 1 to 16) were screened for the presence of dengue specific IgM as described in Fig. 2. The presence of dengue specific IgM in the patients sera was detected using biotinylated anti human IgM monoclonal antibodies, alkaline phosphatase-conjugated streptavidin, and NBT/BCIP substrate. Sera of confirmed negative patients were used as control (lane C).

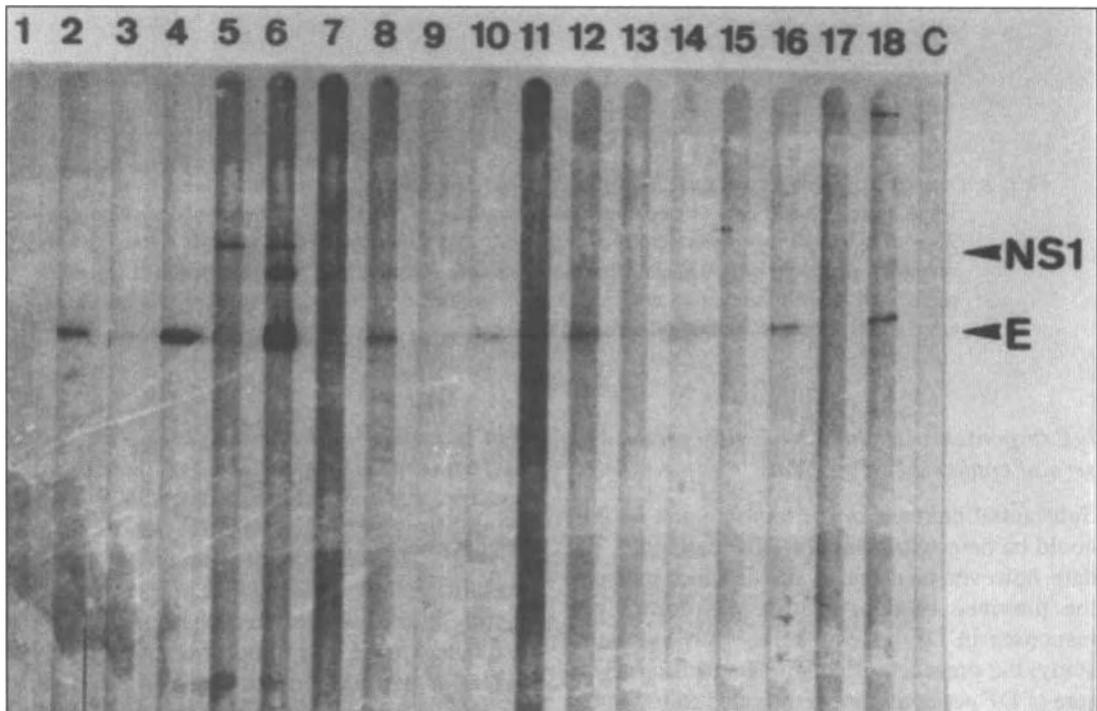


FIG. 6: Detection of IgM specific against dengue 2 NS1 viral proteins. Acute- (lanes 1, 3, 5, 7, 9, 11, 13, 15, 17) and convalescent- (lanes 2, 4, 6, 8, 10, 12, 14, 16, 18) phase sera of DF patients were screened for the presence of dengue specific IgM as described in Fig. 2. The negative patient serum did not show any detectable proteins (lane C).

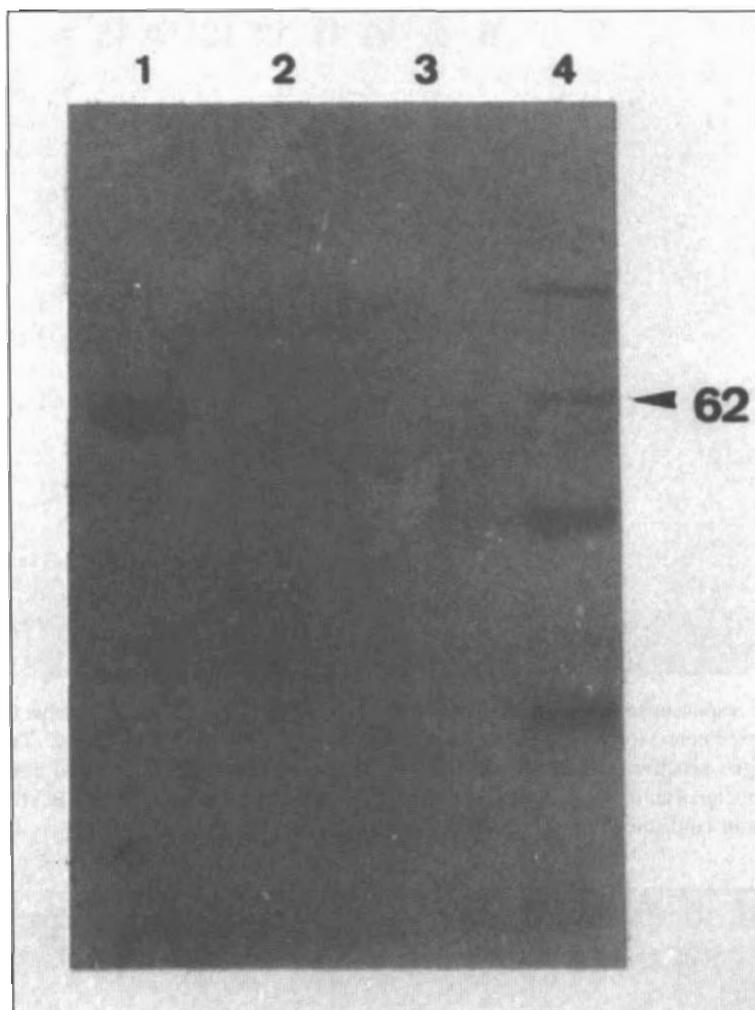


FIG. 7: Detection of IgE specific against dengue 2 NGC viral proteins. Pooled DF patients' sera (lanes 1 and 3) and pooled dengue negative sera (lane 2), were evaluated for the presence of dengue 2 specific IgE. Dengue 2-infected (lanes 1 and 2) and mock-infected cell lysates (lane 3) were prepared, separated by PAGE, and immunoblotted as described in Fig. 1. Detection of IgE was performed using biotinylated anti human IgE monoclonal antibodies, alkaline phosphatase-conjugated streptavidin, and NBT/BCIP substrate.

IgE responses to dengue 2 NGC viral proteins in sera of confirmed DF patients

Substantial increase in the total amount of IgE could be detected in sera of DHF patients. To date, however, no data is available which indicate the presence of dengue protein specific IgE responses in DF patients sera. In the present study, the presence of dengue 2 specific IgE in sera of DF patients was investigated following a similar protocol as described above. Biotinylated human IgE specific monoclonal antibody was used to detect the IgE. Initial screening using pooled secondary DF patients' sera suggested

that IgE specific for the dengue 2 NGC E was present (Fig. 7, lane 1). The IgE response was specific, since no IgE was detectable in pooled sera of healthy dengue negative donors (Fig. 7, lane 2). In addition, it was noted that IgE specific against the dengue 2 E was detectable mainly in the convalescent-phase sera (Fig. 8). IgE bound to the NS1 dimer was detected also in at least two of the convalescent-phase sera (Fig. 8, lanes 7 and 11) and one acute-phase serum (Fig. 8, lane 10). These results suggested that dengue virus infection elicited detectable level of dengue virus specific IgE response especially during the convalescent-phase of the infection.

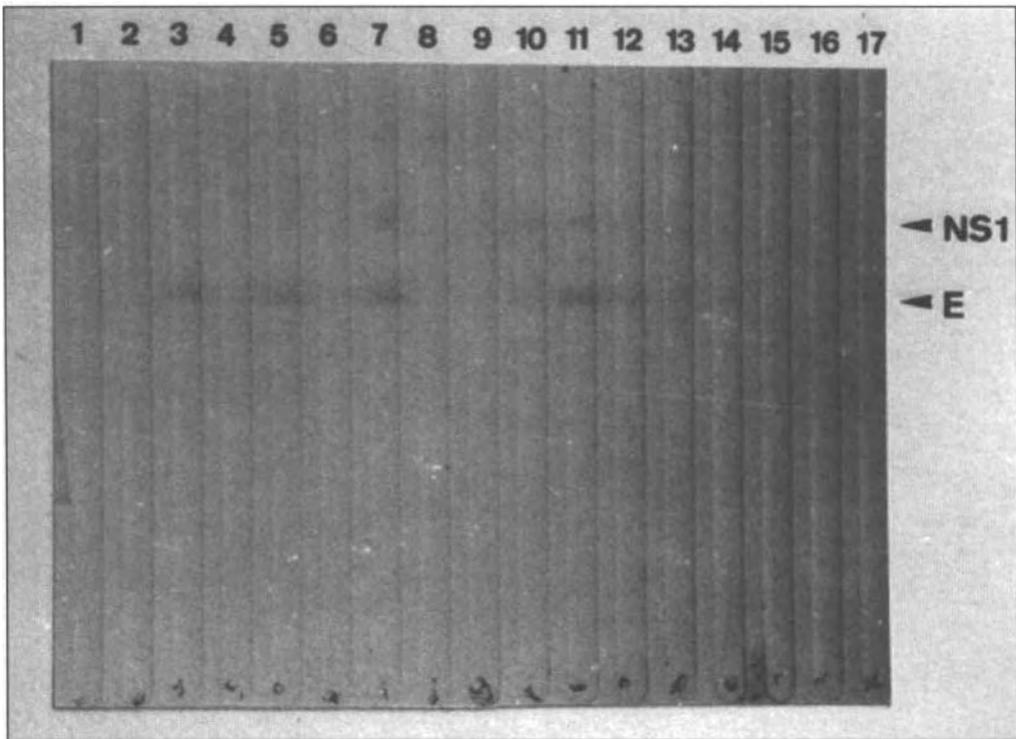


FIG. 8: Detection of **IgE** specific against dengue 2 NS1 viral proteins. Convalescent- (lanes 1,3,5,7,9,11,13,15) and acute- (lanes 2,4,6,8,10,12,14,16) phase sera of dengue negative (lanes 1 and 2; 15 and 16) and DF patients (lanes 3 to 14) were screened for the presence of dengue specific **IgE** as described in Fig. 2. The negative patients' sera and samples to which no serum was added (lane 17) did not show any detectable proteins.

DISCUSSION

The overall objective of this investigation was to determine the dengue 2 NS1 viral proteins that are recognized by DF patients' sera. Using our experimental protocols, it was found that most DF patients' sera had antibodies recognizing the dengue 2 E, suggesting that this protein is highly antigenic. This finding concurs with other previously reported findings.^{8,9} In our study, **IgG**, **IgM** and **IgE** antibodies against the E were present in patients' sera during acute- and convalescent-phase of infection with greater prominence in the later. **IgG** and **IgM** recognizing the dengue 2 NS1 were detected also in patients' sera from which dengue 3 was isolated. This is not surprising, however, since cross-reacting antibody against at least the dengue E has been reported previously.¹⁰ In primary infection, where the **IgM** predominates, substantial presence of **IgM** against the E and NS1 was noted but the **IgG** specific against E, not surprisingly was only faintly detectable. On the other hand, in secondary infection the

opposite was true. The presence of **IgM** against the E and NS1 was faintly detectable, whereas, the presence of **IgG** against these proteins was very prominent. This later observation support the earlier suggestion that the presence of high level of **IgG** tends to interfere with **IgM** binding not only in the ELISA assay¹¹ but also in immunoblotting assay.⁸

Our finding that **IgE** specific for dengue 2 NS1 E was detectable in the convalescent-phase sera of patients with secondary infection concurs with previously reported findings which demonstrated a significant increase in the amount of total **IgE** in sera of DHF patients: and the presence of specific **IgE** response in mice immunized with dengue 2 virus.¹² The potential involvement of **IgE** response in manifestation of severe dengue virus infection has been postulated.¹³ Furthermore, increased level of histamine in blood¹⁴ and release of urinary histamine¹⁵ with increasing severity of dengue infection has also been noted, which argue favorably for the potential significance of **IgE** response in dengue virus infection.

Aside the E, the NS 1 as noted above and the **PrM** were the only other dengue 2 NGC proteins that were recognized by the DF patients' sera. Prominent presence of **IgG** against the NS1 and **PrM** was noted in the convalescent-phase sera of patients with secondary infection. **IgM** and **IgE** recognizing the NS1 were noted also in sera of several patients especially in the convalescent-phase sera, but the levels of detectability were low in comparison to the detection of the E. Unlike the **NS1**, however, the presence of antibody against the dengue 2 NGC **PrM** was detected only in the convalescent-phase sera. No **IgM** or **IgE** responses against this protein could be detected. While results obtained in our study suggest that most secondary DF patients sera had antibodies against the **PrM**, Churdboonchart *et al.*,⁸ reported that only about 20% of DHF/DSS patients had antibody against the protein. One possible reason for this seemingly contradictory finding is that Churdboonchart *et al.*,⁸ had mistaken the **PrM** for C. This is because they did not have the privilege of determining the C using dengue C specific monoclonal antibody, thus, the protein they identify as C, in which most patients with secondary infections had antibody against, is actually the **PrM**. The other possibility is that DF patients' sera have no **IgG** that recognize the C whereas DHF/DSS sera have no **IgG** against the **PrM**. Our results obtained from the only paired DHF patients' sera available for the present investigation, seemed to support the notion that there were probably differences in the **PrM** recognition between DF and DHF/DSS patients. Nevertheless, in a separate study (data not included), using cloned dengue 2 NGC C, we could not detect the presence of **IgG** specific against C in sera of dengue patients regardless on whether DF or DHF/DSS sera were used. Thus, it is likely that the **PrM** instead of C is recognized by most DF patients.

The significance of the **PrM** in stimulating protective immune response, however, is still debatable. It is reported that the **PrM** is immunogenic but not protective.¹⁶ Bray and Lai,¹⁷ however, showed that the **PrM** and M of dengue 2 virus stimulate protective immune response in the mouse encephalitis system. Nevertheless, antibody against the **PrM** has also been shown to enhance dengue virus infectivity.¹⁶ Thus, antibody against the **PrM** which appears significantly in the convalescent-phase sera of DF patients could be important for protection and may also contribute towards increased severity of dengue infection under certain

circumstances.

Results presented in this study, using partially purified dengue 2 NGC virus as antigen, suggest that the E, NS1, and **PrM** are immunogenic. The E and to some extent the **NS1** not only stimulate the immune response but could also stimulate detectable hypersensitivity associated response, namely dengue specific Ig E production. In light of the current effort to develop life vaccine against dengue, it is probably worthwhile noting the potential adverse reaction that could result from a heightened response to some of the dengue viral proteins. The significance of an immune response specific against the **PrM** also requires further investigation. As noted in this study, substantial presence of **IgG** against the protein could be detected in most convalescent-phase sera of patients with secondary infection, suggesting that the **PrM** could be involved in stimulation of a protective immune response.

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